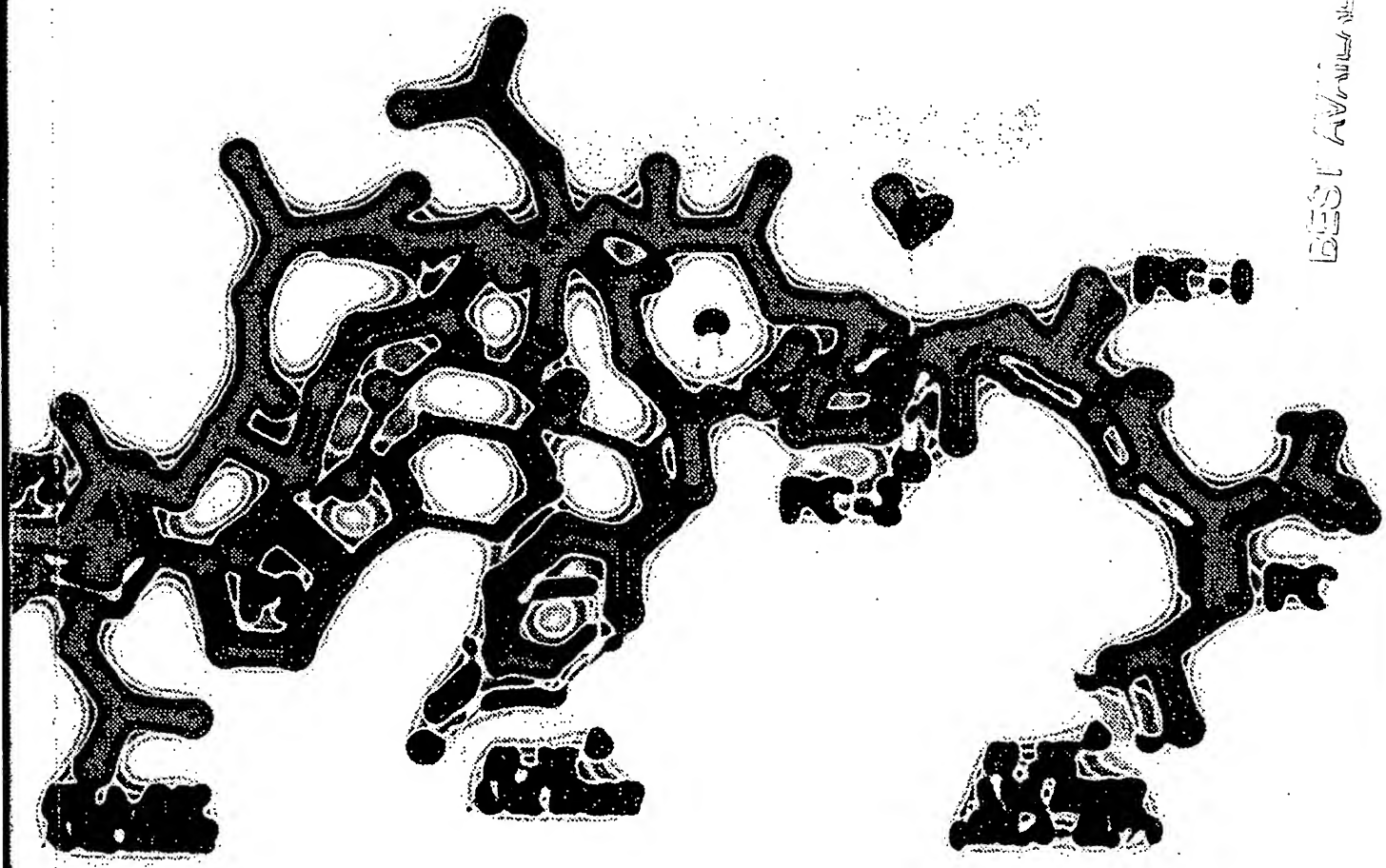
Appendix M

IMMUNOLOGY

CD40, ITS LIGAND AND IMMUNITY



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Structural characteristics of CD40 ligand that determine biological function

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CD40 ligand (CD40L) is a 33 kDa type II glycoprotein which is transiently expressed on the surface of T cells following activation. The demonstration that signals delivered by CD40L are essential for the process of affinity maturation and immunoglobulin isotype switching following antigenic challenge came from the study of X-linked hyper-IgM patients whose T cells cannot express functional CD40L. While some of the biological activities of CD40L, especially on B cells, can be mimicked by monoclonal antibodies (MAb) specific for CD40, it is becoming increasingly clear that CD40L also mediates various functional effects on other cell types. Not only are there distinctions between the activities of CD40L and CD40 MAb, but the manner in which CD40 is ligated appears to play an important part in the biological outcome of signaling through this receptor. In this review, we compare and contrast the activities which can currently be ascribed to CD40L and CD40 MAb and consider the role that ligand oligomerization plays in CD40-mediated signal transduction.

Key words: CD40L / CD40 / NGFR / TNFR family / B cells / Ig secretion

CD40 IS A 50 kDa membrane glycoprotein expressed predominantly on B cells, monocytes, dendritic cells, thymic epithelium and certain carcinomas.[1-3] It is a member of the tumor necrosis factor receptor (TNFR) superfamily,[4,5] a group of related type I transmembrane molecules which, in addition to CD40, includes 60 kDa (p60) and 80 kDa (p80) forms of TNFR, the low affinity nerve growth factor (NGF) receptor, CD27, CD30, OX40, 4-1BB, Fas and TNFR-related protein (TNFR-RP).[6,9] Members of this family are characterized by the presence of multiple cysteine-rich repeats consisting of approximately 40 amino acids in the extracellular amino terminal (Cys) domain.[5] The average sequence homology between family members in the Cys domain is around 25%.

The development of MAb specific for CD40 in the mid-1980s, led to the demonstration that ligation of CD40 could mediate a wide range of activities on human B cells. These include the initiation of homotypic adhesion,[10,11] induction of short-term proliferation in the presence of costimuli,[11,12] and long-term proliferation when CD40 MAb are immobilized on human FcγRII expressed by transfected murine L cells.[13] In addition, CD40 MAb costimulate secretion of IgE in the presence of IL-4[14-16] and other Ig isotypes with IL-2 or IL-10.[17-19] Furthermore, CD40 MAb have been shown to induce bcl-2 expression[20] and rescue germinal center B cells from undergoing spontaneous apoptosis *in vitro*.[21]

Following the cloning of CD40L,[22] it became apparent that the activities ascribed to CD40 MAb mirrored those of a cell surface molecule whose expression, predominantly on activated T cells, is strictly regulated.[23,24] Much of the previously reported help for B cell proliferation and differentiation provided by activated T cells[25-27] could now be explained by the action of CD40L.

In addition to CD40L, TNF and lymphotoxin (LT)-α,[28] ligands for many other receptors in the TNFR superfamily have recently been identified and cloned. These include LT-β,[29] CD27L,[30] CD30L,[31] 4-1BBL,[32] FasL[33] and OX40L (P. Baum *et al*, manuscript submitted). These molecules all have the configuration of type II membrane proteins and exhibit varying degrees of homology with TNF and CD40L. Sequence identity between these ligands is confined to a cysteine-rich region in the C-terminus of the extracellular domain.

TNF and LT-α are known to exist as homotrimers.[34-36] Studies of the crystal structure of LT-α bound to the p60 TNFR have revealed that each receptor monomer binds to a groove formed between two LT-α monomers, each LT-α monomer

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being in contact with two receptor molecules.[37] In this way, trimeric LT-α is able to bind to a trimeric complex of receptors. In addition to their expression as membrane-associated molecules, TNF and FasL, like LT-α, can also exist in soluble forms,[28,33] although it is not clear at the present time whether this represents a common feature shared by other ligands in this family.

In this review, we describe the generation of distinct soluble constructs of the CD40L molecule which differ in their degree of oligomerization. The biological outcome of CD40 ligation using these different forms of CD40L is compared to that mediated by membrane-associated CD40L and CD40 MAb. A clear distinction exists in the functional responses of several cell types to CD40L and CD40 MAb suggesting that the way in which receptor ligation occurs is critical to the subsequent biological consequences of signaling through CD40.

Biochemical properties of soluble CD40L constructs

Given the potent biological activities previously demonstrated for CD40L (reviewed in ref 38), we have generated several distinct forms of the ligand with the aim of developing soluble CD40L molecules which possess comparable biological potency to that of the membrane-associated CD40L. These soluble CD40L constructs were produced in either a mammalian or baculovirus expression system. The first construct to be considered consisted of either the entire extracellular domain or just the TNF-homologous extracellular region of CD40L. The second construct was composed of the hinge-C_H2-C_H3 region of human IgG1 fused to the N-terminus of the entire extracellular domain of CD40L in the configuration of a type II Fc fusion protein.[39] The third soluble CD40L construct contained a modified 33 amino acid leucine zipper motif fused

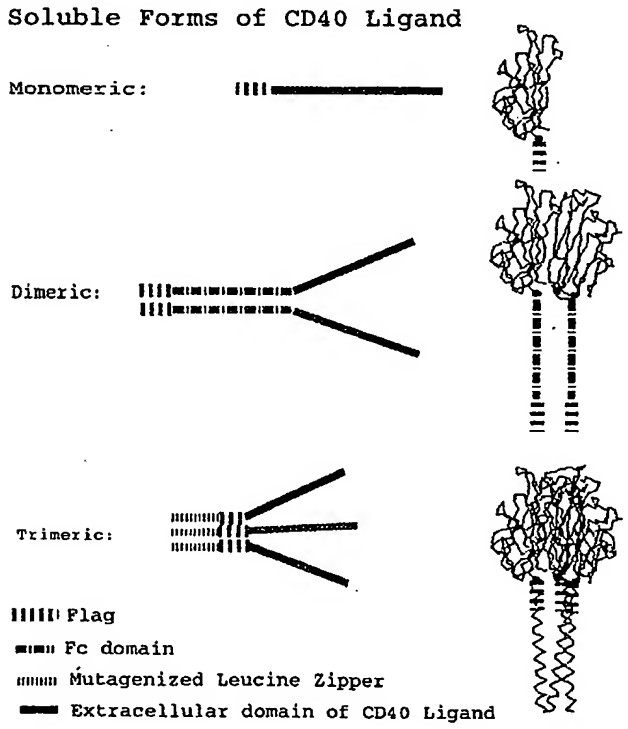

Figure 1. Diagrammatic representations of soluble monomeric, dimeric and trimeric CD40L constructs.

to the N-terminus of the entire extracellular domain of the ligand. Modifications were introduced into the leucine zipper sequence in order to facilitate the formation of trimeric CD40L.[40]

Biochemical and physiochemical studies have revealed the oligomeric nature of these constructs and has confirmed their ability to bind to CD40. The soluble CD40L constructs consisting solely of the extracellular ligand domain were found to migrate on SDS-PAGE at the same M_r under reducing and non-reducing conditions (M_r 30 kDa for the construct containing the entire extracellular domain or 22 kDa for the TNF-homologous form), and are thus referred to as monomeric CD40L. The soluble CD40L construct containing the Fc region of IgG1 (CD40L.FcII) was observed by SDS-PAGE to exist as a 55 kDa protein under reducing conditions, and to be a disulphide-linked homodimer under non-reducing conditions. Gel filtration studies of this dimeric CD40L construct revealed some polydispersity in solution. The CD40L construct containing the modified leucine zipper motif was observed on SDS-PAGE to run as a 35 kDa protein under both reducing and non-reducing conditions. Extensive gel filtration analyses of this construct showed that it existed in solution as a non-covalently linked homotrimer of 120 kDa. Diagrammatic representations of the three soluble CD40L constructs generated are shown in Figure 1. In order to facilitate detection and purification of soluble CD40L, each construct incorporates a short antigenic sequence termed Flag® [41] for which monoclonal antibodies have been generated.

Binding studies have revealed that the monomeric, dimeric and trimeric CD40L constructs are all able to bind to both recombinant soluble CD40 immobilized to a solid phase and native CD40 expressed on the cell surface. Equilibrium binding studies have shown that the dimeric and trimeric forms of soluble CD40L bind CD40 with a similar K_a of approximately $1 \times 10^{10} M^{-1}$. Extensive quantitative binding studies with soluble monomeric CD40L have not yet been performed, but studies which have examined the inhibition of binding of soluble chimeric CD40 Fc fusion protein (CD40.Fc) to membrane-associated CD40L have suggested that the CD40L monomer also binds with high affinity. Thus three distinct forms of soluble CD40L have been generated that bind CD40 with high affinity. As will be discussed, these soluble constructs of CD40L appear to display a hierarchical capacity to trigger biological responses through CD40 which is directly related to their oligomeric status.

Biological consequences of CD40 ligation on B cells

It is now well established that activated human B cells can be induced to proliferate in response to nanomolar concentrations of soluble CD40 MAb.[42,43] In the absence of a costimulus, CD40 MAb in a soluble form are unable to drive resting B cells into cell cycle, although B cell proliferation can be induced if CD40 MAb are immobilized on human FcγRII expressed by transfected L cells.[13] Stimulation through CD40 in this manner presumably mimics the signal delivered to B cells by membrane-associated CD40L.[22,24] F(ab)$_2$ fragments of CD40 MAb are also costimulatory for B cells but have a somewhat reduced activity compared to whole antibody.[43,44] In contrast, Fab fragments of CD40 MAb are not only unable to costimulate B cell proliferation, but act to antagonize stimulatory signals delivered by intact antibody.[44] We have generated two CD40 MAb, M2 and M3, which have been shown previously to completely inhibit the binding of CD40L to soluble CD40.Fc.[45] The addition of Fab fragments of the M2 CD40 MAb to cultures containing anti-IgM-activated human B cells inhibited proliferation induced by both whole M2 antibody (Figure 2A) and soluble trimeric CD40L (Figure 2B). The inhibitory effect of Fab M2 on proliferation induced by CD40L was comparable to that seen in the presence of CD40.Fc.

The ability of both F(ab)$_2$ fragments and whole CD40 MAb to costimulate B cell proliferation while Fab fragments of the same antibody are non-stimulatory, suggests that crosslinking of receptors on the B cell surface is a prerequisite for entry into cell cycle following CD40 ligation. This is supported by the observation that purified soluble CD40L in a trimeric form is a potent costimulus for B cell proliferation, while a monomeric construct of the ligand is not (Figure 2C). CD40L expressed as a membrane-associated molecule is directly mitogenic for B cells in the absence of a costimulus.[22,24] While Fab fragments of M2 are effective inhibitors of proliferation induced by membrane-associated CD40L, it is of interest that under these culture conditions whole M2 antibody has a comparable antagonistic effect (Figure 2D). Furthermore, in contrast to nearly all CD40 MAb, in the absence

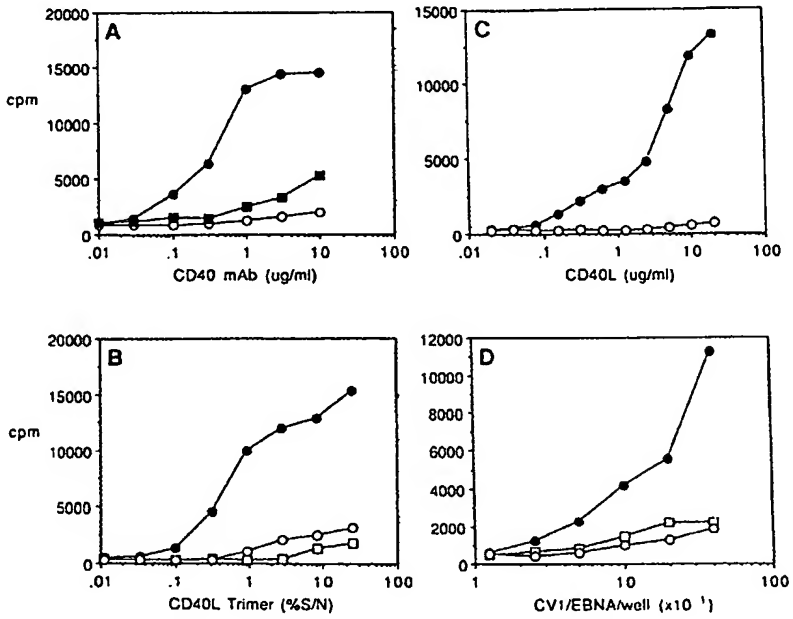

Figure 2. Induction of B cell proliferation by different forms of CD40L and CD40 MAb. Purified tonsil B cells (1×10^5/well) were cultured for 3 days in the presence (panels A-C) or absence (panel D) of 5 μg/ml anti-hIgM immobilized on beads and the following additions. (A) Fab fragments of M2 CD40 MAb (○) or whole M2 in the presence (■) or absence (●) of 20 mg/ml Fab M2. (B) soluble human trimeric CD40L alone (●) or in the presence of 10 μg/ml CD40. Fc (□) or 20 μg/ml Fab M2 (○). (C) purified soluble murine trimeric (●) or monomeric (●) CD40. (D) CV1/EBNA cells expressing membrane-associated human CD40L alone (●) or in the presence of 20 μg/ml Fab M2 (○) or 10 μg/ml whole M2 (□). All our results are expressed as the mean cpm tritiated-thymidine incorporation from triplicate cultures.

of a costimulus, both membrane-associated and soluble trimeric CD40L can induce increased expression of CD23, B7-1 (CD80) and HLA class II.[38] CD40L-mediated induction of these molecules is inhibited by both whole M2 antibody[45] and Fab fragments (R.J. Armitage, unpublished observations). Therefore, soluble CD40 MAb, which induce B cell proliferation in the presence of a costimulus can also act to antagonize the direct stimulatory action of CD40L.

Although monomeric CD40L is unable to induce B cell proliferation, it can both bind to CD40 and induce increased expression of class II molecules (W.C. Fanslow, unpublished observations), an activity shared with soluble trimeric and membrane-associated CD40L. Recently, Hasbold and Klaus described what they term an abortive pathway of CD40 signaling in CBA/N mice.[46] These animals carry the X-linked immunodeficiency defect *xid* which results from a point mutation in the gene encoding the B cell specific tyrosine kinase Btk,[47,48] which is also defective in X-linked agammaglobulinemia (XLA) in man.[49,50] Binding of soluble CD40L or MAb specific for murine CD40 failed to costimulate proliferation of CBA/N B cells but could induce both CD23 and class II expression. These observations, together with the contrasting activities of different forms of CD40L and CD40 MAb on normal B cells, point to the existence of distinct CD40-mediated signaling pathways, activation of which is determined by the manner in which CD40 is ligated. One pathway involves the signaling cascade of which Btk is a part, where crosslinking of CD40 is essential to induce proliferation, while another pathway, independent of Btk activity, mediates early events in cellular activation including the induction of CD23 and class II expression, and possibly that of B7. Our data suggest that activation of this Btk-independent pathway does not require crosslinking of CD40 but probably relies instead on a ligand-induced conformational change of the receptor.

Regardless of whether different multimeric and multivalent forms of CD40L and CD40 MAb directly activate B cells, in all cases addition of costimuli such as anti-IgM or IL-4 significantly enhance proliferation mediated through CD40. In contrast, such costimuli do not enable monomeric CD40L or Fab fragments of CD40 MAb to induce proliferation.

A further example of a common activity for CD40L and CD40 MAb on B cells comes from studies which have examined the regulation of apoptosis. Both CD40 MAb and CD40L rescue germinal center B cells from undergoing spontaneous apoptosis in culture.[21,51] Similarly, apoptosis of Burkitt's lymphoma (BL) cell lines induced by the crosslinking of surface IgM is inhibited by both ligand and antibody.[51,52] Interestingly, in the absence of surface IgM crosslinking, both CD40L and CD40 MAb deliver an inhibitory signal to BL cells resulting in a significant reduction in the rate of proliferation.[53] This effect of CD40 MAb is augmented by immobilization of the antibody. When human B cell lymphomas were transferred into mice with severe combined immune deficiency (SCID), the *in vivo* treatment of mice with CD40 MAb inhibited the growth of lymphoma cells, increasing the survival of the animals.[53] Furthermore, CD40L and CD40 MAb share the ability to enhance proliferation of myeloma cells through the induction of autocrine IL-6 production, a finding which is compatible with the observation that malignant plasma cells from myeloma patients express CD40.[54]

While clear differences exist between CD40L and CD40 MAb in the requirement for a costimulus to induce B cell proliferation, the binding to CD40 of ligand or antibody alone in any form is insufficient to cause Ig secretion. Whether human B cell stimulation through CD40 is instigated by membrane-associated or soluble multimeric CD40L,[22,24,55] or by immobilized or soluble CD40 MAb,[14-17] the presence of IL-4 is essential for secretion of IgE and IgG4. Conversely, either IL-2 or IL-10 can act as costimuli with CD40L and CD40 MAb to induce secretion of IgM, IgG1, IgG2, IgG3 and IgA from human blood and tonsil B cells.[17-19,55] Studies with surface IgM$^+$ B cells isolated from X-linked hyper-IgM (HIGM) patients, whose T cells are unable to express functional CD40L,[56] have suggested that IL-10, but not IL-2, may act as a switch factor for several IgG isotypes.[56,57] In contrast, IL-2 probably acts to promote secretion of these isotypes from B cells which have already undergone isotype switching *in vivo*.

Compatible with the findings that monomeric CD40L and Fab fragments of CD40 MAb are unable to costimulate B cell proliferation, these forms of ligand and MAb are unable to costimulate secretion of any Ig isotype in the presence of IL-2, IL-4 or IL-10 (R.J. Armitage, unpublished observations). The comparative abilities of different forms of CD40L and CD40 MAb to elicit B cell responses is summarized in Table 1.

Biological consequences of CD40 ligation on non-B cells

In addition to its range of activities on B cells, CD40L has stimulatory effects on other cell types. Human and murine T cells and $\gamma\delta^+$ thymocytes activated with CD3 MAb, PHA or ConA are induced

Table 1. Summary of B cell responses induced by different forms of CD40L and CD40 MAb

Stimulus	Activation*	Proliferation		Differentiation‡
		– costimulus	+ costimulus†	
Membrane CD40L	+ +	+ + +	+ + + +	+ + + +
Soluble trimeric CD40L	+ +	+ +	+ + + +	+ + + +
Soluble dimeric CD40L	+	+	+ + +	+ + + +
Soluble monomeric CD40L	+	–	–	–
Immobilized CD40 MAb§	+	+ +	+ + + +	+ + + +
Soluble CD40 MAb	+¶	–	+ + +	+ + + +
Fab CD40 MAb	nd‖	–	–	–

*Activation defined by some or all of the following: increased cell size, enhanced expression of CD23, class II or B7-1 (CD80).
†Costimuli include anti-IgM, IL-4, CD20 MAb, phorbol ester, SAC.
‡Secretion of IgE with IL-4 as costimulus, IgM, IgG and IgA with IL-2 or IL-10 as costimuli.
§CD40 MAb are most efficiently immobilized on human FcγRII (CD32) expressed by transfected murine L cells.
¶Only a minority of CD40 MAb induce activation in the absence of costimuli.
‖Not determined.

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HUTNFA: SPVAHVVANPQAEG---QLQWLNRRANALLANGVELRD----NQLVVPSEGLYLI
HCD40L: QTAAHVISEASSKTT-SVLQWAEKGYYTMSNNLVTLENG---KQLTVKREGLYYI
MCD40L: QLAAHVVSEANSNAA-SVLQWAEKGYYTMSNLVMLENG---KQLTVKREGLYYV
SHEET*: SPVAHVVA-----*-------------ALLAN*-ELR-----NQLVV-**GLYLI

HUTNFA: YSQVLFKGQGC-P---STHVLLTHTISRIAVS-YQTKVNLLSAIKSPCQRETPEG
HCD40L: YAQVTF----CSNREASSQAPFIASLCLKSPG--RFERILLRAANTHSSAK-PCG
MCD40L: YTQVTF----CSNREPSSQRPFIVGLWLKPSI--GSERILLKAANTHSSSQ-LCE
SHEET*: YSQVLFKGQ**-*---*---VLLTHTISRI--*---TKVNLLSAIKS--------*

HUTNFA: AEAKPWYEPIYLGGVFQLEK----GDRLSAEINRPDYLDFA-ESGQVYFGIIAL-
HCD40L: QQ------STHLGGVFKLQP----GASVFVNVTDPSQVSHG--TGFTSFGLLKL-
MCD40L: QQ------SVHLGGVFELQA----GASVFVNVTDASQVIHR--VGFSSFGLLKL-
SHEET*: -----WYEPIYLGGVFQL-*-----*ERKSAEIN-------*****QVYFGIIAL-
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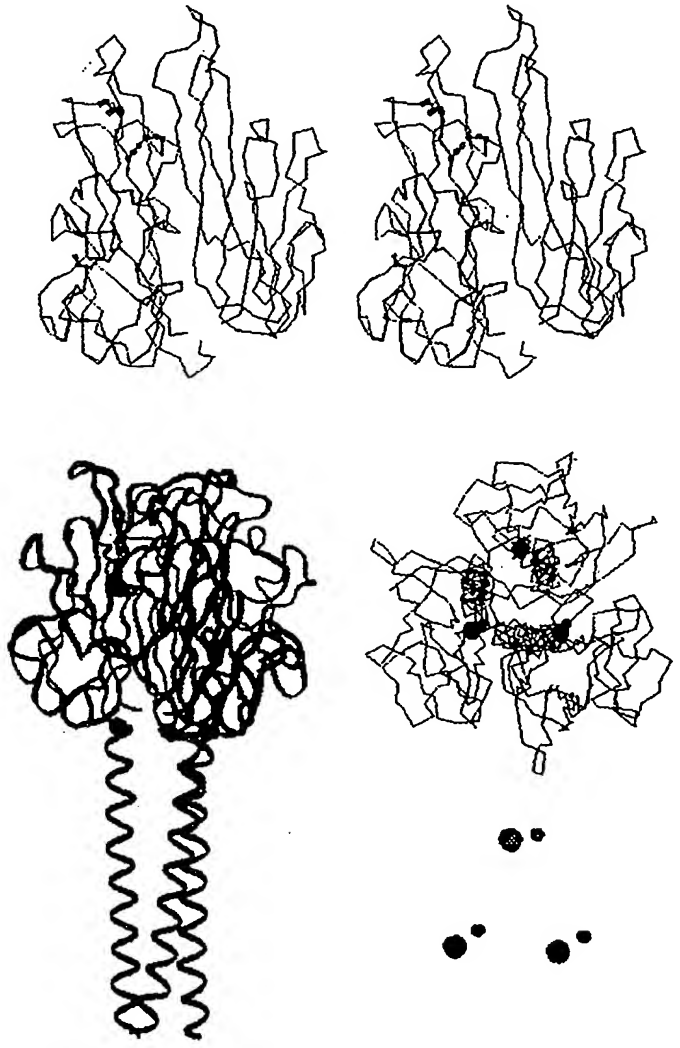

Figure 3. Top: Sequence alignment between the CD40L folding domain and TNF-α used in the generation of models of CD40L. Middle: Stéreoscopic view of a model of CD40L (green) superimposed on one of the subunits of trimeric TNF-α (red) based on its crystal structure. Shown in blue is a second subunit of TNF-α as found in the trimeric form. Disulfides are shown in ball and stick representations in the respective colors with sulfur atoms in yellow. Bottom, Left: A model of CD40L with the engineered trimeric leucine zipper at the N-terminus of the ligand molecule. Right: Highlights of the compatibility between the C-terminus of the trimeric leucine zipper (small spheres) with the N-terminus of the CD40L trimer (large spheres).

to proliferate and secrete cytokines in response to CD40L.[58-60] In contrast to their costimulatory activities on B cells, soluble CD40 MAb have no such effect on T cells. However, if CD40 MAb are immobilized on the culture vessel, they provide as potent a costimulatory signal as that delivered by CD40L.[61] It appears, therefore, that a higher degree of CD40 crosslinking or a more sustained signal may be required to induce T cell proliferation, compared to that sufficient to drive B cells into cell cycle.

CD40 is present at low levels on peripheral blood monocytes, and its expression is greatly enhanced following exposure to IL-3, GM-CSF or IFN-γ.[3] In combination with these cytokines, CD40L can induce monocytes to secrete IL-6, IL-8 and TNF. Furthermore, in the absence of any costimulus, CD40L is a potent inducer of macrophage tumoricidal activity. However, in contrast to most CD40L activities on B and T cells, the functional effects of CD40L on monocytes cannot be mimicked by CD40 MAb, presented either in a soluble or immobilized form. In fact, soluble M2 CD40 MAb is as effective as CD40.Fc at antagonizing the stimulatory signal delivered to monocytes by CD40L.[3]

Recent studies using cell lines derived from Hodgkin's lymphoma (HD) patients have demonstrated a similar functional distinction between binding of ligand and antibody to CD40. Membrane-associated and soluble trimeric CD40L both induced IL-8 secretion and significantly enhanced production of IL-6, TNF and LT-α from HD lines with a 'T cell like' or 'B cell like' phenotype.[62] In addition, CD40L enhanced expression of B7-1 and CD54 and reduced the level of CD30 expressed by these cell lines. Soluble or immobilized CD40 MAb had no such stimulatory effect on these cell lines. In fact, soluble CD40 MAb were able to inhibit CD40L-enhanced cytokine production and alteration of surface-antigen expression on HD lines in a manner reminiscent of its inhibitory effects on CD40L-stimulated monocytes.

Requirements for signaling through other receptors in the TNFR family

In order to better understand the requirement for crosslinking in CD40-mediated signal transduction, it is informative to consider the biological consequences of receptor ligation for related members of the TNFR family. In addition to expressing CD40, B cells can express p80 TNFR, CD27, 4-1BB and Fas. Proliferation of activated B cells can be induced by MAb specific for p80 TNFR and, to a lesser extent by antibodies directed to Fas (R.J. Armitage, unpublished observations). However, in contrast to the activity of soluble CD40 MAb, antibodies specific for TNFR and Fas will only costimulate B cell proliferation if presented in an immobilized form. As a soluble trimeric molecule, TNF has been reported to have costimulatory activity for B cell proliferation,[63] although, as with CD40L, it appears that TNF has a more potent effect on B cells when it is presented in a membrane-associated form.[64] In contrast to TNFR MAb, soluble or immobilized antibodies directed against CD27 and 4-1BB have no discernible effect on human B cell proliferation (R.J. Armitage, unpublished observations).

When one considers the biological effects of TNFR family-specific MAb on T cells, a common picture emerges. Just as CD40 MAb in an immobilized, but not soluble, form will costimulate proliferation of activated T cells,[61] so too will MAb specific for TNFR, CD27, CD30, 4-1BB and Fas.[31,65-68] This pattern of activity of TNFR family MAb reflects the previously reported T cell costimulatory effects of the appropriate membrane-associated ligands.[30-32] Where examined, the same MAb directed against these TNFR family members are, in a soluble form, antagonistic toward the stimulatory effects of immobilized anti-receptor antibodies or membrane-associated ligands (M.R. Alderson, unpublished observations).

It appears likely, therefore, that for maximal signal transduction a requirement for receptor crosslinking, which brings receptor molecules into close proximity with one another, is a common feature shared by all members of the TNFR family.

Can the biological activity of CD40L be explained by its predicted structure?

Although the ligand binding regions of CD40 and TNF receptor show significant sequence homology, the receptor binding domains of CD40L and TNF only show weak sequence identity. However, the consensus sequence derived from multiple alignment between the members of this family of ligands suggests a similar tertiary structure for all members.

Using the crystal structure of TNF as a template, a three-dimensional model of CD40L can be generated using FOLDER, a distance geometry based homology modeling software.[69]

However, in the predicted model for CD40L (Figure 3, top panel) the only possible disulfide linkage between cysteines cannot be fashioned on the template of TNF. This is due to insertions and deletions, relative to the TNF sequence, on either side of the two cysteines in the CD40L molecule. In CD40L these cysteines are present at the tail end of D strand and at the beginning of G strand, whereas in TNF the cysteines are in the loop between D and E strands and at the tail end of F strand. In the CD40L model, a standard geometry for the disulfide linkage cannot be achieved without perturbing the backbone atoms near the beginning of the G strand as shown in Figure 3 (middle panel). Since, in the trimeric configuration, the G strand of one subunit interacts closely with the F strand of the other neighboring subunit, disulfide bonding between cysteines in the soluble form of CD40L might contribute to the partial destabilization of the trimeric ligand.

TNF, in its soluble form, is known to exist as homotrimers.[34,36] The fact that monomeric constructs of the TNF-homologous extracellular region of CD40L display only a very limited biological activity compared to trimeric CD40L, suggests that CD40L monomers, unlike TNF, do not readily associate in solution. In order to offset the potential destabilizing effect of the disulfide linkage on trimeric forms of CD40L, we used a trimeric leucine zipper[40] as a stem to hold three CD40L extracellular domains in an orientation similar to the trimeric form of TNF deduced from its crystal structure (shown in Figure 3, bottom panel). The Cα atoms of the N-terminal residue in the CD40L model are geometrically compatible to the Cα atoms of the C-terminal residue of the designed stem. The coordinates for the mutagenized leucine zipper were generated using the trimeric stalk of influenza hemagglutinin as a template.[70]

Discussion

CD40L mediates a range of biological activities on normal B cells including the initiation of early activation events such as induction of activation-associated surface antigens,[38] entry into cell cycle[22,24,71,72] and, in combination with the appropriate cytokines, secretion of Ig.[55,73] While many of these activities are shared also by CD40 MAb, it is noteworthy that the ability of CD40L to induce CD23, B7 and class II expression, and to drive B cells into cell cycle in the absence of any costimulus, is not a feature generally mimicked by most CD40 antibodies. The ability of different soluble and immobilized forms of CD40L and CD40 MAb to induce B cell proliferation (summarized in Table 1) appears to depend on the degree of oligomerization or valency of these reagents, demonstrating a hierarchy in the signaling potency of such molecules. So, although the crosslinking of CD40 on the B cell surface is essential to induce proliferation, the manner in which this process occurs significantly affects the outcome of receptor ligation. In the absence of costimuli, membrane-associated CD40L provides the strongest proliferative signal for B cells, while soluble trimeric CD40L and immobilized CD40 MAb are somewhat less stimulatory. Dimeric CD40L alone has relatively weak activity while soluble CD40 MAb exhibit little, if any, proliferative effect. However, in the presence of costimuli, CD40L and CD40 MAb have comparable activity for the induction of B cell proliferation and differentiation, while monomeric CD40L and Fab fragments of CD40 MAb, being unable to crosslink CD40, are inactive. The comparable activity of CD40L and CD40 MAb for B cell differentiation in the presence of costimuli may be related, at least in part, to the ability of some costimuli to enhance CD40 expression and thus the degree of receptor crosslinking.

In an attempt to explain the differences in the proliferative activity of CD40L and CD40 MAb in the absence of costimuli, it is possible to construct a model based on the results of studies on the crystal structure of LT-α complexed with the p60 TNFR.[37] Here, each receptor chain is bound to a contiguous epitope formed by two subunits of the ligand, whereas each ligand molecule is bound to two receptor chains by two discontiguous epitopes. Two molecules of ligand provide a full binding epitope for one receptor chain, whereas one ligand molecule provides partial binding epitopes for two receptor chains. In order to provide full binding epitopes for two or three receptor chains, as observed in the crystal structure of the ligand-receptor complex, three ligand molecules are required. If the dimeric form of the ligand is

considered as a trimer with a missing subunit, it could provide a full epitope for one receptor chain and partial epitopes for two other receptor chains. Thus in this model, dimeric forms of such ligands would be predicted to have a biological activity somewhat less potent than that of trimeric ligand. In contrast, bivalent CD40 MAb, which binds to only two receptor molecules would be less active than dimeric ligand.

Although the LT-α/TNFR modeling predicts that CD40 may exist as a homotrimer on the cell surface, this has yet to be proven. However, biochemical studies have shown that interchain disulphide bond exchange between CD40 monomers can occur both on the cell surface[74] and in solution,[39] supporting the notion of the formation of higher ordered oligomers. In both these cases, however, CD40 dimers, and not trimers, were detected. Full occupancy of each CD40 molecule is provided by contact of approximately 550 Å^2 with each of two CD40L molecules. If CD40 can associate as a trimer, the total area of contact with trimeric CD40L would be 3300 Å^2. However, if two associated CD40 molecules are sufficient for biological signaling, a trimeric ligand would offer 1100 Å^2 contact area for each receptor chain whereas a dimeric ligand would offer 1100 Å^2 for one CD40 molecule and 550 Å^2 for the other.

The greatest distinction between the activities of CD40L and CD40 MAb is seen when CD40 is ligated on non-B cells. Both membrane-associated CD40L and immobilized CD40 MAb are potent costimuli for T cell proliferation[58-61] while soluble CD40L, in comparison, has only a weak effect and soluble CD40 MAb are inactive.[61] Binding of immobilized ligand and antibody may well provide for a greater degree of crosslinking and lead to a prolonged period of signaling compared to that delivered by soluble CD40L or CD40 MAb which could be rapidly internalized or shed from the cell surface.

Both membrane-associated and soluble CD40L provide a strong costimulatory signal to monocytes and HD cell lines resulting in the secretion of multiple cytokines.[3,62] CD40 MAb are unable to mimic this activity whether presented in an immobilized or soluble form. At present, it is not clear why these cell types fail to respond to CD40 MAb when they clearly express CD40 and can be stimulated by CD40L. One possibility is that the interaction of CD40L with its receptor results in a unique conformational change in CD40 on these cell types which is essential for one type of signal transduction pathway and cannot be imitated by binding of antibody.

As with the CD40L-CD40 interaction, studies of other related ligand-receptor pairs have pointed to receptor crosslinking as an obligatory common feature shared by members of the TNFR family for successful signal transduction. However, the fact that soluble MAb directed against Fas are able to induce apoptosis appears, initially, to be anomalous to this model. In fact, the soluble Fas antibodies which have been shown to be apoptotic are either of an IgM isotype (i.e. pentameric),[75] or are IgG3 MAb,[76] an isotype which is prone to aggregation. In contrast, Fas-specific MAb of an IgG1 isotype will only trigger through Fas if they are immobilized.[68] In a soluble form, these MAb can antagonize signals delivered by Fas-specific antibody of an IgM isotype. The observation that soluble forms of FasL have comparable apoptotic activity for Fas$^+$ target cells as that seen for membrane-associated FasL,[33] suggests that soluble FasL may well associate into homotrimeric complexes, in a similar way to that demonstrated for soluble TNF and predicted for CD40L, and thus have the capacity to effectively crosslink its receptor. The studies with Fas MAb present one further anomaly. In light of the isotype-related differences observed in the activity of soluble Fas MAb, why are IgM CD40 MAb no more stimulatory in a soluble form than those of other isotypes?[77] Clearly, in addition to the common mechanisms of signal transduction shared by this family of ligands, more subtle distinctions in their activities remain to be elucidated.

Soluble LT-α has been shown to form heterotrimers with membrane-associated LT-β.[29] Whether other ligands in the TNF family can form similar hetero-complexes remains to be determined. However, if such associations are common, it could provide a means by which the range of functional activities of each ligand is amplified. Similarly, the association of different receptors in the TNFR family with one another, or with other currently undefined molecules, could provide a model which would help in our understanding of the factors governing the common and unique biological activities already described for some of these ligands.

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